Ultrastructural Studies of a Compatible Interaction Between *Xanthomonas campestris* pv. *malvacearum* and Cotton

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ABSTRACT

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The ultrastructural changes in leaves and cotyledons of the susceptible cotton line Acala 44 inoculated with *Xanthomonas campestris* pv. *malvacearum* were studied for up to 6 days postinoculation. Early changes included formation of vesicles between the plasmalemma and cell wall, followed by disappearance of the granal and stromal membranes of the chloroplasts. Degeneration of mitochondria occurred after ultrastructural alteration in the chloroplasts. The last cellular organelles to undergo structural degeneration were the nucleus and plasmalemma. Fibrillar material was present at the external cell surfaces near bacteria at 5 days postinoculation. By day 6 many cell walls were broken and fragmented. Bacteria had entered the mesophyll cells, and specific organelles could no longer be distinguished. Large accumulations of electron-dense fibrillar material were present in the intercellular spaces at 6 days postinoculation, when water-soaking symptoms were apparent.

Bacterial blight caused by *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye is an important disease of upland cotton (*Gossypium hirsutum* L.) (3,15). A histological study by Thiers and Blank (20) revealed bacteria surrounded by slime in intercellular spaces of infected cotton leaves. The bacteria invaded both spongy mesophyll and palisade cells. A light microscopic investigation of the blight-susceptible cotton line Acala 44 (Ac 44) (5) showed no structural defense reaction by the host plant against *X. campestris* pv. *malvacearum*. A later study (9) showed that final bacterial populations in compatible leaves of Ac 44 were several orders of magnitude higher than the final populations in leaves of the blight-immune line.

Ultrastructural studies of compatible interactions between bacterial phytopathogens and other crops such as apple, potato, tobacco, and bean have been reported (10,11,16,17). The only published ultrastructural study of bacterial interactions with cotton (6) was primarily concerned with the incompatible interaction and was restricted to the first 24 hr postinoculation. No ultrastructural changes were observed in the compatible host Ac 44 during that period. In this study we monitored ultrastructural changes in leaf and cotyledon cells and in bacteria for 6 days following inoculation of host tissue with *X. campestris* pv. *malvacearum*. A preliminary report of this work has been published (1).

MATERIALS AND METHODS

Host growth environment. Ac 44, a breeding line which contains no major genes for resistance to bacterial blight (3), is susceptible to infection by all 18 races of *X. campestris* pv. *malvacearum*. Acid-delinted seeds were grown in 15-cm-diameter clay pots filled with a commercially prepared mix of peat moss and vermiculite (Jiffy Mix Plus; Jiffy Products of America, West Chicago, IL 60185). Plants were kept in a greenhouse with a daily maximum temperature of 32 $\pm$ 3 C and night temperature of 20 $\pm$ 3 C. Relative humidity was 100% at night, while the daily mean humidity was 59%. Seedlings had fully expanded cotyledons 2 wk from germination, and at 3 wk

the second and third foliage leaves were expanded. The fully expanded young leaves, as well as full expanded cotyledons, were used in this study.

Methods of inoculation and bacterial culture. A highly aggressive strain of *X. campestris* pv. *malvacearum* race 3 was used as inoculum at 10^6 bacteria per milliliter. It was isolated in 1978 by W. M. Johnson from a cotton plant in a field at Altus, OK, and identified as race 3 using a standard set of differentials (14). The maintenance and growth of bacteria followed the method of Essenberg et al (9). Cotyledons were inoculated with bacteria suspended in a sterile, saturated solution of $CaCO_3$ using a sterile hypodermic syringe. The syringe without a needle was pressed gently against the abaxial leaf surface and inoculum of 10^6 bacteria per milliliter was injected until complete water-soaking of the cotyledon was observed. Foliage leaves were vacuum infiltrated by using the procedure of Essenberg et al (9). Two controls each were used for cotyledons and leaves. One control was infiltrated with saturated $CaCO_3$ solution, the other control was infiltrated with distilled deionized water. These procedures introduced inoculum doses of $0.6-1.0 \times 10^4$ bacteria per square centimeter of leaf area. Bacterial population levels were determined by dilution plate counting. Population determinations were made each day from the same inoculated leaves used for electron microscopy (EM). Leaves which had been stored for 7 yr were also examined. These leaves had been harvested and dried after developing confluent water soaking.

Tissue preparation for EM. Leaf segments (~ 1 mm^2) were fixed every 12 hr for 6 days with 0.1 M potassium phosphate-buffered 4% glutaraldehyde (pH 7.3) at 4 C for 2 hr. Tissues were subsequently washed and postfixed with 2% osmium tetroxide for 4 hr, dehydrated in a graded series of water-ethanol solutions, and embedded in the firm-formulation epoxy resin of Spurr (18)

Silver-reflective thin sections were cut with a Sorvall MT-2 ultramicrotome and a diamond knife. Sections were collected on uncoated grids and stained with 0.5% uranyl acetate and 0.4% lead citrate (21), and were examined with an RCA EMU-3G electron microscope at 100 kV.

RESULTS

Bacterial populations increased logarithmically in leaves for 3 days postinoculation and stabilized at approximately 10^6 bacteria per square centimeter, as previously reported (9).
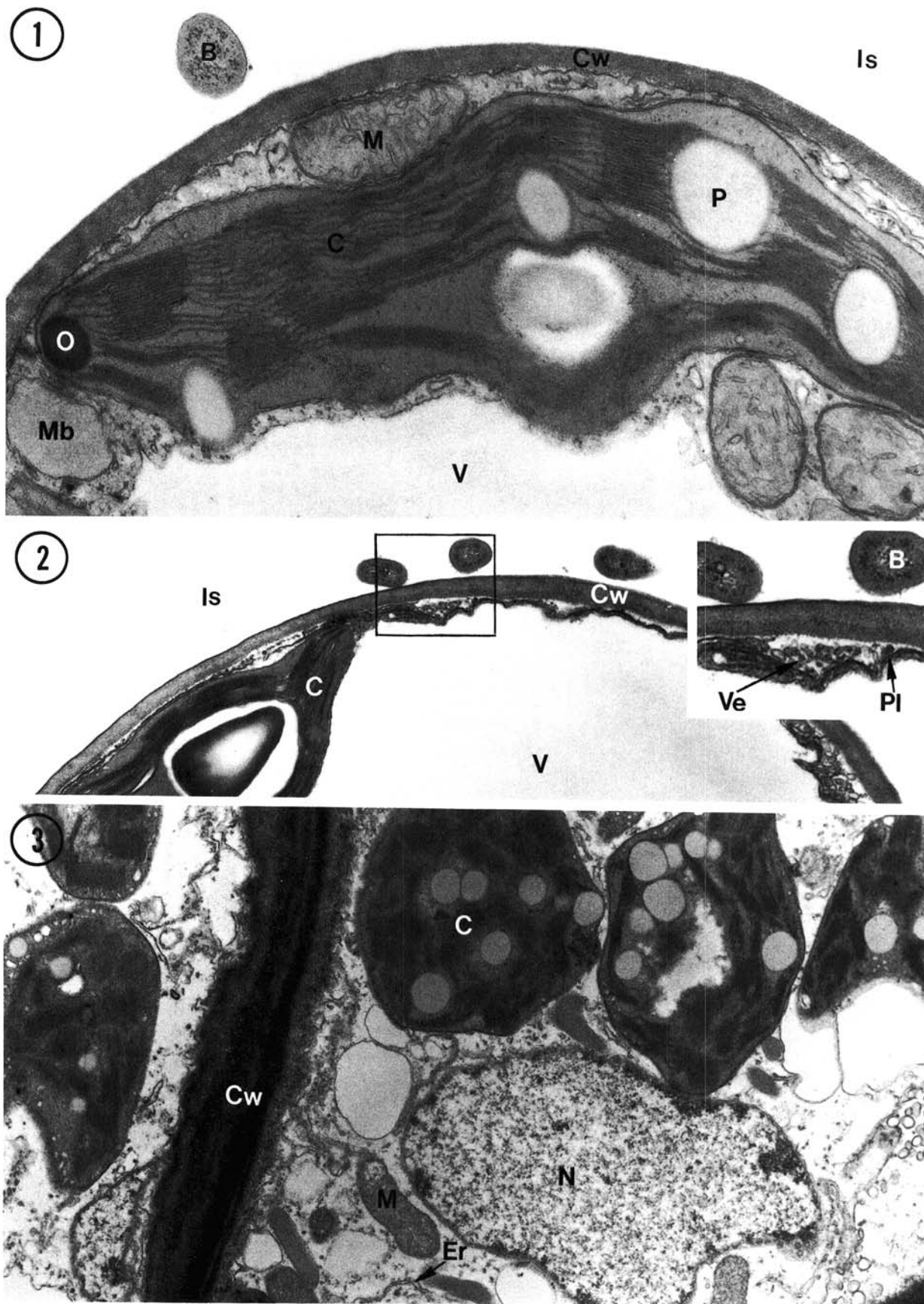
Figs. 1–3. 1, Twelfth hour postinoculation with *Xanthomonas campestris* pv. *malvacearum*, cotton foliage leaf mesophyll cell. Cell structure and cytoplasmic components appear as in control, bacterium is close to cell wall (×20,400). **2,** Forty-eighth hour postinoculation foliage leaf mesophyll cell. Vesicles are present between plasmalemma and cell wall, and three bacterial cells are close to cell wall (×19,700). Note (inset) higher magnification of vesicles (×39,440). **3,** Seventy-second hour postinoculation cotyledon mesophyll cell. Chloroplasts appear round while mitochondria and nucleus appear unaltered (×12,150). B = bacterium, C = chloroplast, Cw = cell wall, Er = endoplasmic reticulum, Is = intercellular space, M = mitochondrion, Mb = microbody, N = nucleus, O = osmiophilic granule, P = plastoglobule, Pl = plasmalemma, V = vacuole, and Ve = vesicles.
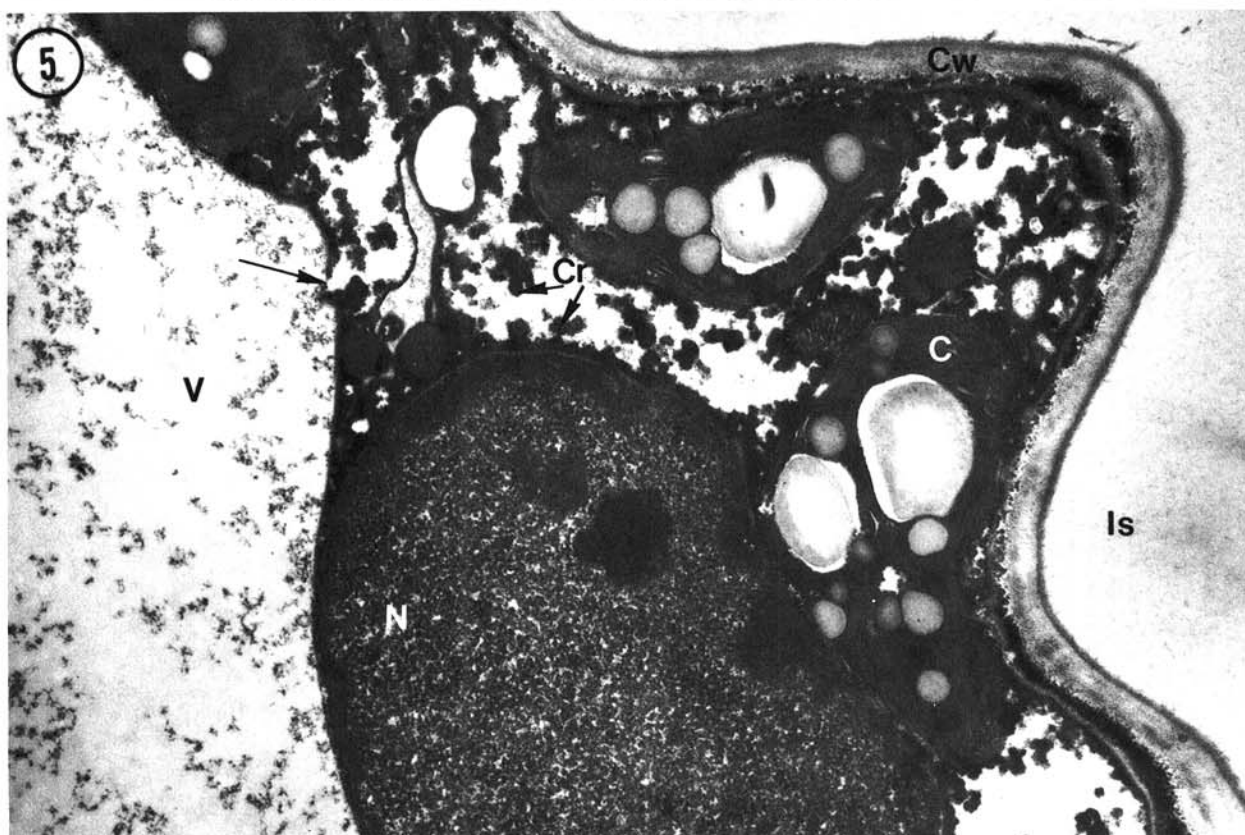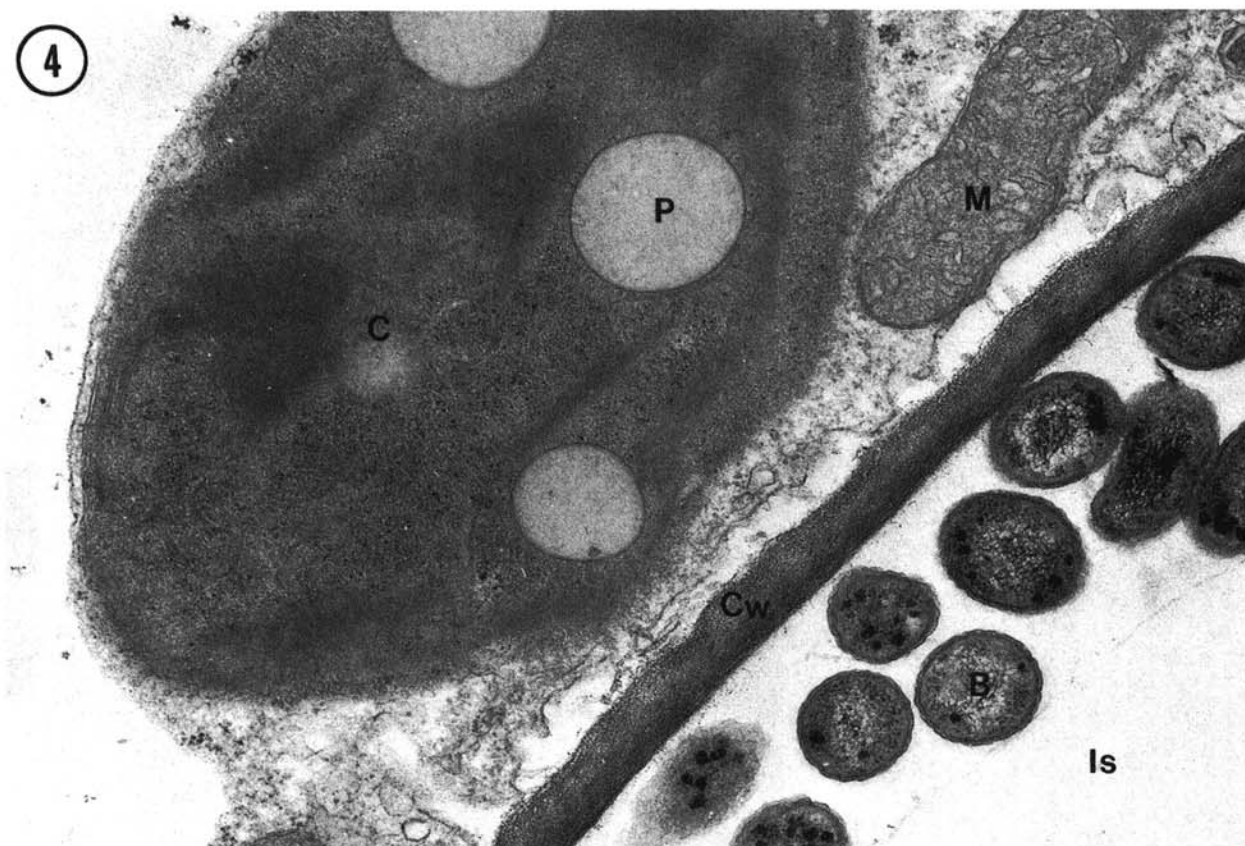
Figs. 4–5. 4, Ninty-sixth hour postinoculation, cotyledon mesophyll cell. Chloroplast appears round and has lost most of its membranes, mitochondrial membranes appear normal (×29,000). **5,** Fifth day postinoculation, foliage leaf mesophyll cell with coagulated ribosomes, dense nucleus and chloroplast. Tonoplast membrane is ruptured (arrow) (×15,600). B = bacterium, C = chloroplast, Cr = coagulated ribosomes, Cw = cell wall, Is = intercellular space, M = mitochondrion, N = nucleus, P = plastoglobule, V = vacuole.
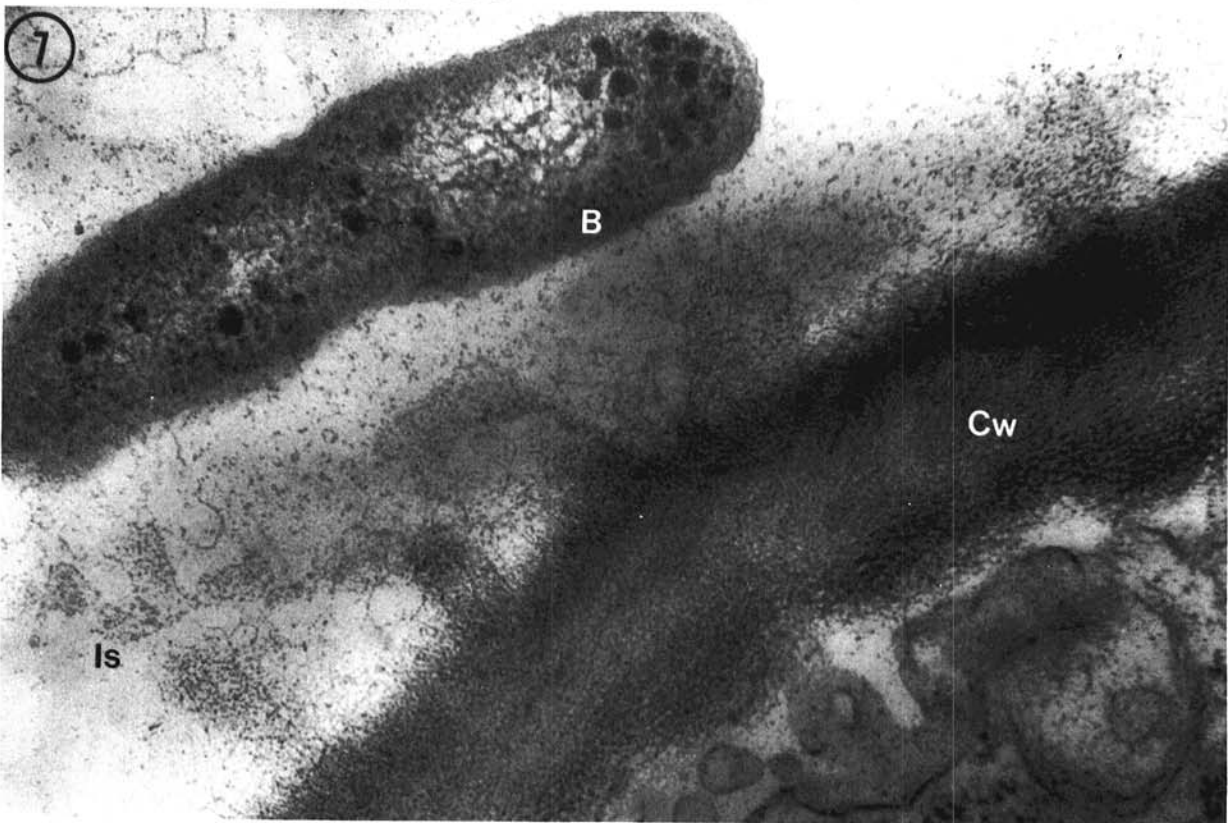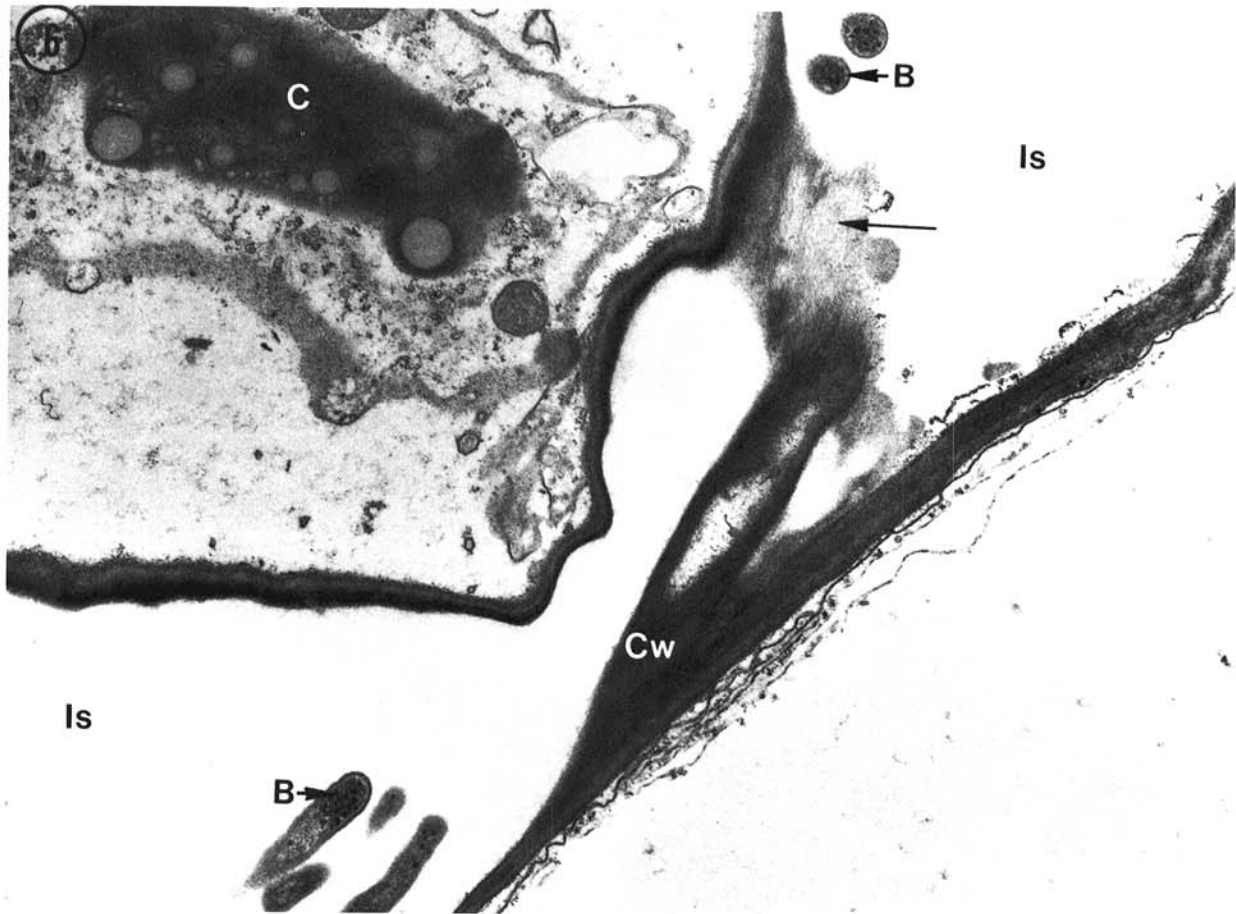
**Figs. 6–7. 6,** Fifth day postinoculation, cotyledon mesophyll cells. Note loosening of middle lamella (arrow) (×11,500). **7,** Fifth day postinoculation, cotyledon mesophyll cell with apparent loosening in outer cell wall near dividing bacterium (×63,500). B = bacterium, C = chloroplast, Cw = cell wall, Is = intercellular space.
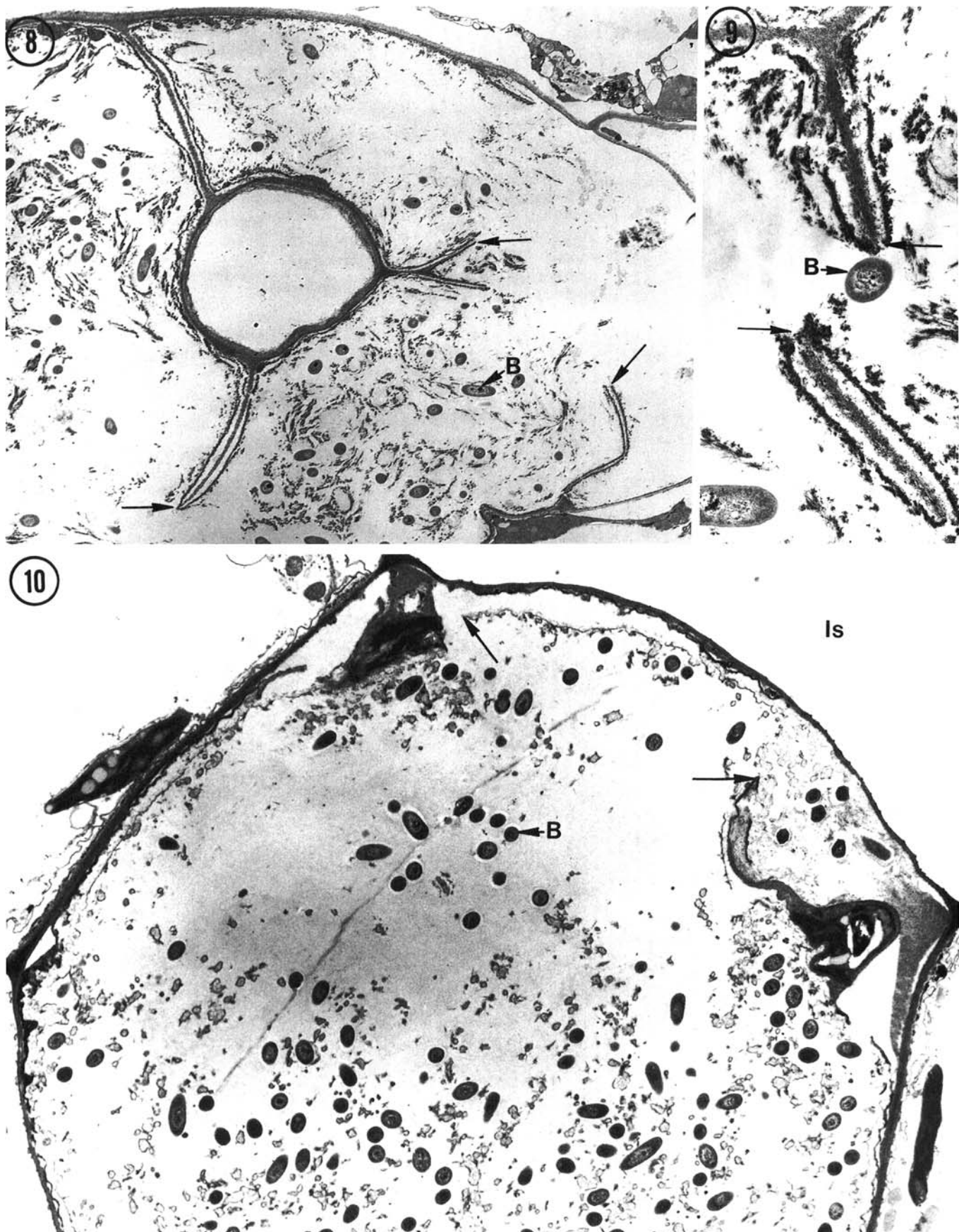
Figs. 8–10. 8, Sixth day postinoculation, cotyledon mesophyll cell. Note broken host cell walls (arrows) (×3,150). **9,** Sixth day, cotyledon mesophyll cell. Note broken wall at higher magnification (arrow). Electron-dense fibers appear to separate from cell wall (×14,300). **10,** Sixth day, cotyledon mesophyll cells with bacterial cells inside host cell. Note broken plasmalemma (arrow) (×7,000). B = bacterium, Is = intercellular space.
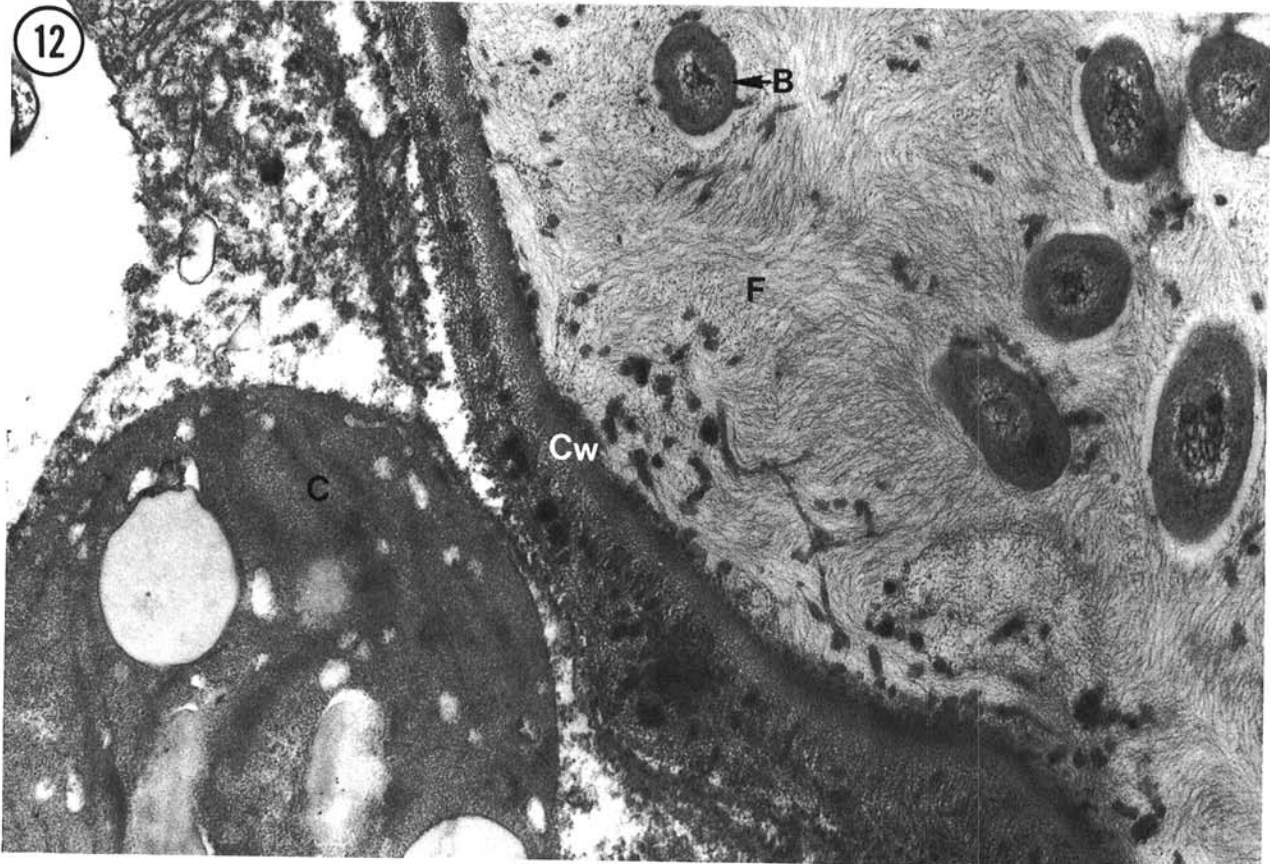
Figs. 11–12. 11, Sixth day postinoculation, foliage leaf showing four mesophyll cells with fibrillar material filling the intercellular spaces (×9,200). **12,** Sixth day, higher magnification of foliage leaf mesophyll cell with fibrillar material in the intercellular space. Bacterial cells are surrounded by clear capsular material (×35,500). B = bacterium, C = chloroplast, Cw = cell wall, F = fibrillar material, Is = intercellular space.
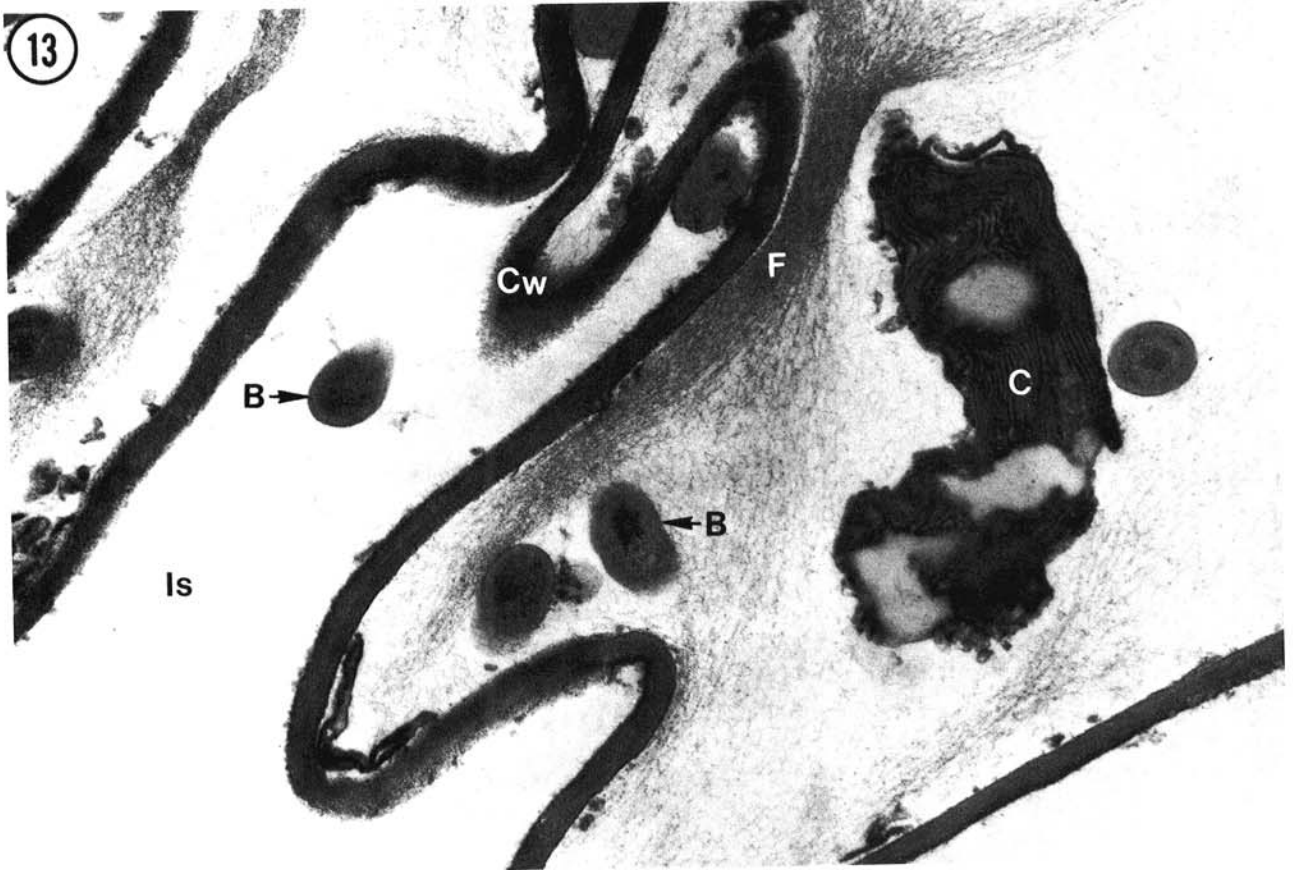
Figs. 13–14. 13, Sixth day postinoculation, foliage leaf mesophyll cells with fibrillar material and bacteria inside mesophyll cell (×35,000). **14,** Seven-year-old, dried, diseased leaf showing dense fibrillar material (×39,800). B = bacterium, C = chloroplast, Cw = cell wall, F = fibrillar material, Is = intercellular space.

A similar sequence of ultrastructural changes was apparent in both cotyledons and leaves that had been inoculated with *X. campestris* pv. *malvacearum*, whereas no progressive degenerative changes were observed in the two types of controls. On the first day postinoculation, bacterial cells were observed in the intercellular spaces (Fig. 1) of leaf sections in which cell organelles and their membrane systems were indistinguishable from those of the control sections.

At two days postinoculation some vesiculation was seen between the plasmalemma and cell wall (Fig. 2). Plasmolysis was also observed in many cells. At 3–4 days postinoculation the outer membranes and granal and stromal lamellae of chloroplasts were not intact and chloroplasts had become rounded. Outer mitochondrial membranes and cristae could still be observed (Figs. 3 and 4). The nucleus appeared relatively unaffected, while rough endoplasmic reticulum cisternae appeared swollen (Fig. 3). At this stage, cell membranes were disrupted and showed vesiculation and irregular outlines. Plastoglobuli within the chloroplasts increased in number as the disease progressed.

Just before final degeneration (5–6 days postinoculation) chloroplasts were irregular in shape with broken outer membranes. Tonoplasts were ruptured and organelles were dispersed throughout the cell (Figs. 5 and 6). Ribosomes were often coagulated (Fig. 5). Nuclei appeared more electron-dense with indistinct outer envelopes (Fig. 5). Intercellular spaces were expanded due to dissolution of middle lamellae and walls of adjacent cells (Fig. 6). At day 5 postinoculation fibrillar material appeared to have loosened from host cell walls (Fig. 7) in close apposition to the bacteria. This was followed by breaks in cell walls (Figs. 8 and 9) and entry of bacteria into mesophyll cells (Fig. 10). At this stage no organelles were distinguished within the broken plasmalemma.

The cells of infected leaves did not degenerate at equal rates. At any time during the week following inoculation, cells in several states of degeneration were visible in the same tissue. However, the sequence of changes in each cell was always as described above (Figs. 1–10). Some fibrillar material surrounding bacteria was seen inside and outside of mesophyll cells (Figs. 11–13). At the same time, 5–6 days postinoculation, intact leaves started to show the water-soaking symptom. Very densely stained aggregates of similar fibrillar material were seen in micrographs of dried diseased leaves many years postinfection (Fig. 14).

DISCUSSION

The compatible interaction between *X. campestris* pv. *malvacearum* and *G. hirsutum* was characterized by severe damage to the membranes of all organelles, as well as to wall structure of mesophyll cells. Granal and stromal lamellae of chloroplasts were the earliest membranes altered. The most persistant structures were the nucleus and the plasmalemma. Early membrane destruction of chloroplasts and eventual disruption of other host cell membranes have been observed during compatible host-bacterial pathogen interactions (7,10,11,17). Since the ultrastructural changes in host membranes reported here are similar to changes that occur during leaf senescence (4), they may be due to autolysis of the host in response to the pathogen.

In this compatible system there were no structures enveloping bacteria at host cell wall surfaces such as were observed in the incompatible reaction between cotton and *X. campestris* pv. *malvacearum* (2). However, a breakdown of plant cell wall did occur. This was observed to begin with a loosening of fibrillar material in the outer layer of cell wall (Figs. 6 and 7) as observed in other compatible interactions (7,13,16,23–25). Wall loosening was followed by dissolution of the rest of the cell wall (Figs. 8 and 9), leaving fibrillar material throughout the cell. *X. campestris* pv. *malvacearum* is known to produce pectinmethylesterase, polygalacturonase, and cellulase (12,22) which may be responsible for the damage to host walls observed here. The invasion of cotton host cells by *X. campestris* pv. *malvacearum* (Fig. 10) was first reported by Thiers and Blank (20). Invasion of xylem parenchyma has been observed in a bacterial disease of vascular tissue (23).

Invasion of bean leaf cells by *Pseudomonas phaseolicola* has been reported (19), but was not observed in other studies of bean leaves following inoculation with *P. phaseolicola* (17) and *P. syringae* (7).

Dense fibrillar material was seen inside and outside of the cells at late stages of the disease (Figs. 11–14). Wallis and co-workers have observed somewhat similar fibrillar material in vascular tissue of cabbage (24) and tomato (23) infected with bacterial pathogens. They interpreted the material they saw as shredded host cell wall. Since the fibrillar material in infected cotton cotyledons and leaves was always associated with bacteria, we favor the hypothesis that it is bacterial exopolysaccharide slime (EPS), a substance which is produced so abundantly in this disease that it sometimes oozes onto surfaces of leaves (20). That EPS can stain as intensely as the fibrillar material we observed was demonstrated when El-Banoby and Rudolph (8) made electron micrographs of bean leaves inflitrated with purified EPS from *P. phaseolicola*. Identification of the fibrillar material must await further chemical characterization. Since fibrillar material appears late in the disease when host cells are dying and collapsing, it may be part of a medium that enables the bacteria to resist unfavorable environmental conditions. Usually by the time fibrillar material was seen, bacterial cells had developed a capsular coating.

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